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14. ABSTRACT

The overall objective of the research program is to develop allosteric modifiers of hemoglobin that are effective *in vivo*. The ability of a novel agent 02-50, a trifluoromethyl substituted analog of LR16, was found to strongly modulate the P_{50} value of purified hemoglobin. 02-50 displayed activity greater than that of LR16. However, the presence of human serum albumin (HSA) strongly modulated the pharmacological properties of LR16 and 02-50 agents. Experiments with denatured HSA also revealed that the albumin interactions with LR-16 were not specific in nature and both LR-16 and 02-50 are lipophilic and capable of diffusing from LEH particles.

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FINAL TECHNICAL REPORT

Department of the Navy
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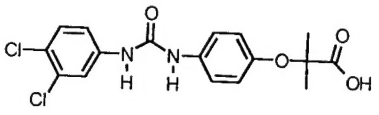
SUMMARY

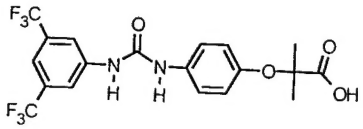
The overall objective of the research program is to develop allosteric modifiers of hemoglobin that are effective *in vivo*. Allosteric modifiers of hemoglobin have potential uses in extending the storage time of red blood cells as well as in the development of a liposome-encapsulated hemoglobin (LEH)-based artificial blood substitute. Because the prevention of oxidation to hemoglobin and membrane components is a major requirement for the successful long-term storage of red blood cells and hemoglobin-based blood substitutes, modulation of the oxygen content of the samples of interest using allosteric modifiers of hemoglobin may be of merit in minimizing unwanted oxidative processes during storage. The ability of a novel agent to modulate the oxygen binding properties of hemoglobin have been compared with the known compound LR16. A 3,5-di(trifluoromethyl) substituted analog of LR16 (compound 02-50) was found to strongly modulate the P_{50} value of purified hemoglobin. 02-50 displayed activity greater than that of LR16. The P_{50} values of liposome-encapsulated hemoglobin suspensions (containing human hemoglobin stripped of 2,3-diphosphoglycerate) were increased from 10 mm Hg to P_{50} values of 32 mm Hg and 61 mm Hg, respectively, using 02-50 concentrations of 0.75 mM and 1.5 mM, respectively. However, the presence of human serum albumin (HSA) strongly modulated the pharmacological properties of LR16 and 02-50 agents. 1 mM LR16 shifts the P_{50} of hemoglobin free in solution from 8 mm Hg to a value of 49 mm Hg; however, the addition of physiologically relevant concentrations of 50 mg/ml HSA right shift the oxygen dissociation profile of hemoglobin to control P_{50} values (8 mm Hg). Strong modulation was observed for both lipid-encapsulated hemoglobin and unencapsulated hemoglobin. The effects of LR16 and 02-50 in liposome-encapsulated hemoglobin suspensions were significantly reduced by the presence of HSA. HSA is well known to have two highly specific binding sites for lipophilic and negatively-charged drugs that are dependant upon the native conformation of the protein. We used a fluorescence anisotropy titration assay to study the intrinsic fluorescence of an an agent known to interact in a highly specific manner with the HSA binding pockets. We then conducted competition binding experiments to see how LR16 competes with this fluorophore for the binding pockets. Whereas the well known HSA-binder ibuprofen interacted with HSA in a highly specific manner, LR-16 showed reduced competition. The reduced competition displayed by LR-16 suggests that its binding to the blood protein is much less specific or in a different site relative to the ibuprofen binding site. Experiments with denatured HSA also revealed that the albumin interactions with LR-16 were not specific in nature and that protein denaturation did not markedly attenuate drug binding. Taken together the data indicate that LR-16 binding is nonspecific and may be difficult to reduce by subtle structure changes to the drug molecule. High sensitivity differential scanning calorimetry studies revealed that both LR16 and 02-50 interact with the lipid bilayers and induce significant changes in the bilayer phase transitions. The calorimetric data indicate both LR-16 and 02-50 are lipophilic and ready interact with bilayers. The lipophilic nature of the agents likely facilitate diffusion out of the LEH particles, especially when the LEH particles are dispersed in blood and surrounded by albumin. These type of lipid bilayer interactions are unwanted for LEH particle production in that they facilitate drug transport from the particle and drug binding by albumin which drives the equilibria for more drug departing the particle. Molecular modeling studies showed negatively-charged O2-50 fits in the deoxy-hemoglobin DPG binding pocket in between several positively charged amino acids of the two beta-chains. Several H-bonds between O2-50 and positively charged amino acids (His 2; Lys 82; His 143) of beta -chains are possible. O2-50 may stabilize the deoxy-hemoglobin by binding to the two beta-chains, thereby decreasing the oxygen affinity of hemoglobin. Future analog development studies will focus on structural changes which conserve the allosteric modulation of oxygen affinity will reducing drug associations with the HSA blood protein.

INTRODUCTION

Pharmacological approaches to the optimization of the oxygen affinity of liposome-encapsulated hemoglobin (LEH), a potential blood replacement fluid, have been studied. In our recent studies we have researched interactions of LR-16 and 02-50, two potent allosteric modifiers of human hemoglobin, with human serum albumin (HSA), lipid bilayers, and human hemoglobin. The structures of LR-16 and 02-50 are shown below.



LR-16



02-50

EXPERIMENTAL METHODS

Preparation of Purified Human Hemoglobin Solutions. Outdated blood was centrifuged at 4 °C at 1000 x g for 15-20 min. The supernatant was discarded, and the packed cells were carefully resuspended (i.e. without vigorous shaking) in an equal volume of cold 0.15 M NaCl and recentrifuged at 1000 x g. The 0.15 M NaCl washing was repeated four times.The packed, washed cells in heavy glass centrifuge tubes were lysed by addition of an equal volume of cold purified water, followed by addition of chloroform (5% of the total volume). The mixture was stirred for 30 min at 4 °C and then centrifuged at 3000 rpm for 10 min at 4 °C to remove the major portion of cell debris and chloroform-containing viscous phase. The supernatant containing hemoglobin was centrifuged for 30 min at 4 °C and 9000 x g. The supernatant containing hemoglobin was removed leaving behind the remaining cell debris. Organic phosphates were removed by dialysing extensively at 4 °C against 0.5 mM TES buffer containing 0.1 M NaCl, pH 7.5. The dialysis buffer was initially changed at one hour intervals for 6 hours followed by every 4-6 hours for next 24 hours. Finally, ionic impurities were removed by passing the hemoglobin solution through a column of Dowex MR-3 mixed bed ion exchange resin that was washed and pre-equilibrated with Millipore purified water at 4 °C. The purified hemoglobin solution was concentrated two-fold using Amicon Centriprep concentrators by centrifuging at 3000 x g and 4 °C and stored at -20 °C.

Drug Dissolution. Drug stock solutions of 3 mM or less in 2-{[tris-(hydroxymethyl)-methyl]-amino}-ethanesulfonic acid (TES) buffer (0.05 M TES, 0.14 M NaCl, pH 7.40) were prepared by vigorous vortexing. The mixture was sonicated briefly and/or warmed to 40 °C if the dissolution process was slow as evidenced by a lack of optical clarity of the solutions. Finally, pH was adjusted to 7.40 if needed. LR16 stock solutions of >0.02 M were prepared by initially dissolving in TES buffer at pH 10-11 and then adjusting the pH to 7.40 with HCl. Stock

solutions were also prepared in DMSO for the competition binding experiments.

P_{50} Determination. Recording of curves of equilibrium binding of oxygen to hemoglobin or LEH was carried out with the Hemox Analyzer (TCS Medical Products, Huntingdon Valley, PA). The operating principle of the Hemox Analyzer is based on dual-wavelength spectrophotometry for the measurement of the amounts of oxygenated and deoxygenated hemoglobin and a Clark membrane electrode for the measurement of the oxygen partial pressure. Briefly, an approximately 3 ml hemoglobin solution was drawn into the cuvette which also contained an oxygen sensitive membrane electrode, a thermistor probe for temperature measurement and a magnetic stirring bar. Measurements were carried out at 37 °C. The sample was allowed to equilibrate with air flowing through the solution and the extent of oxygenation was recorded on X-Y recorder as a function of oxygen partial pressure. The P_{50} values were calculated from oxygenation curves. A P_{50} value is defined as the pO_2 value at which 50% oxygen saturation of the sample occurs.

Fluorescence-Based Competition Binding Experiments. The intrinsically fluorescent agent camptothecin (known to interact with HSA in a highly specific manner) was obtained from Boehringer Ingelhem (Lot#95-002). Dimethyl sulfoxide (HPLC grade, Aldrich, Milwaukee, WI) was used to prepare stock solutions of camptothecin at various concentrations, which were stored in the dark at -20 °C. Working solutions of 1.0×10^{-3} M camptothecin carboxylate were prepared by diluting a stock solution of camptothecin in DMSO with PBS buffer at pH values of 10.0. The Sigma Chemical Co. (St. Louis, MO) supplied the human serum albumin (HSA) employed in the binding experiments. A 2.5×10^{-3} M stock solution of HSA was prepared in PBS buffer at a final pH of 7.4. The concentration of the HSA was determined on a weight-to-volume basis (g/L). A Milli-Q UV PLUS purification system (Bedford, MA) was used to acquire high-purity water. For the competition binding experiments, 3.0×10^{-3} M camptothecin carboxylate working solutions were prepared. LR-16 competitive binding interactions were analyzed.

Steady-state fluorescence anisotropy measurements were recorded using a SLM 9850 fluorometer interfaced with an IBM computer. The samples were excited at an excitation wavelength of 370 nm. The excitation monochromator bandwidth was set to 4 nm. Fluorescence emission was isolated from scattered light by utilizing 400 nm long band-pass filters. For the camptothecin carboxylate binding experiments with HSA, ten test tubes of varied HSA concentration were prepared. Volumes of the 2.5×10^{-3} M HSA stock and PBS buffer pH of 7.4 were combined in ten test tubes to create different HSA concentrations ranging from 0.5×10^{-5} M to 1.8×10^{-4} M. The test tubes were placed in a VWR Scientific Waterbath (Model 1235) set at 37 °C for approximately five minutes. Following this, the first test tube was removed and a 5.0×10^{-6} M concentration of the drug was prepared by adding an appropriate volume of the 1.0×10^{-3} M camptothecin (37 °C) working solution to the test tube. The drug and HSA solution was immediately mixed on a Vortex Genie 2 from Fisher Scientific for approximately three to five seconds. Immediately after, the solution was transferred to a thermostatic (37°C) sample cell and the anisotropy measurement was recorded. The same procedure was followed for the remaining nine samples. For each tube, the anisotropy measurement was recorded within one minute upon the addition of the drug. This short acquisition time secured that the anisotropy measurements reflected the initial form of the drug added instead of a lactone-carboxylate equilibrium form.

The procedure followed for the competition binding experiments with LR-16 was very similar to the description above. A 3.0×10^{-3} M camptothecin carboxylate working solution was

prepared and kept at 37 °C. Once the HSA/PBS solutions were prepared, an appropriate volume of a competitor stock was added to each tube. The competitor concentration was identical for all ten test tubes. LR16 competitive binder concentrations of 1.0 x10 $^{-4}$M, 1.0 x10 $^{-3}$ M, 5.0 x 10 $^{-3}$M, 1.0 x 10 $^{-2}$M and 5.0 x 10^{-2}M were studied using the stock solutions discussed earlier. The same competition concentrations for ibuprofen were studied excluding the 1.0 x 10 $^{-2}$ M. Once the competitor was added, the test tubes were placed in the waterbath, like before, and the measurements were taken by employing the same technique described for the HSA binding experiment.

Hemoglobin Modeling Studies. The molecular structure of deoxy-hemoglobin and O2-50 complex was built in QUANTA (Molecular Simulations Inc.) installed on Silicon Graphics workstations. Deoxy-hemoglobin structure was obtained from Protein Databank (PDB entry 2HHB) x-ray crystallography study (G. Fermi; M.F. Perutz; B. Shaanan and R. Fourme). The O2-50 was constructed in QUANTA/Molecular Builder. Standard atom types in the QUANTA package were used for building O2-50 molecule.

High Sensitivity Differential Scanning Calorimetric (DSC) Studies of Allosteric Modifier-Lipid Bilayer Interactions. For the preparation of DMPC liposomal formulations, the following amounts and ingredients were used: 8.34 mg of DMPC, 8.34 mg of DMPC and 0.18 mg of LR-16, and 8.34 mg of DMPC and 0.18 mg of 02-50. The mixtures were placed in 10 ml test tubes, and 2 ml of chloroform was added into each of the tubes. Additionally 0.5 ml of methanol was added into the tubes containing LR-16 and 02-50 (these compounds dissolved poorly in chloroform). Next the solvent was evaporated under a stream of nitrogen gas. Test tubes with the films were kept in the vacuum (at least 12 hours). Prior to the DSC measurement the films was hydrated by adding 2 ml of PBS (pH 7.4, temperature of ~37 °C) followed by high speed vortexing (approx. 5 minutes). In this manner the following suspensions was obtained : 1) 6 mM DMPC liposome suspension (blank); 2) 0.3 mM LR-16 DMPC (6 mM) liposomal formulation; and 3) 0.3 mM 02-50 DMPC (6 mM) liposomal formulation. A Microcal VP-DSC MicroCalorimeter (MicroCal Inc.) was used to determine the calorimetric signature of blank DMPC liposomes as well as LR-16 and 02-50 liposomal formulations. For DSC measurement 0.5 ml of suspension was used, with the reference cell containing 0.5 ml of PBS (pH 7.4). The following experimental conditions was used: scanning range, 5 to 35 C, prescan thermostating, 1 hour, scanrate, 20 deg/hour and high feedback mode. Each sample was scanned twice (to check reproducibility).

RESULTS

A new agent which we synthesized, compound 02-50, was found to display activity greater than that of LR16. At 02-50 concentrations of 0.75 mM and 1.5 mM, respectively, the P_{50} values of LEH preparations containing human hemoglobin stripped of 2,3-diphosphoglycerate were increased from 10 mm Hg to P_{50} values of 32 mm Hg and 61 mm Hg, respectively. Our data indicates that the allosteric modifiers LR16 and 02-50 are capable of diffusing into LEH particles composed of distearoylphosphatidylcholine (DSPC): dimyristoylphosphatidylglycerol (DMPG): cholesterol [molecular ratios of 4:1:3, respectively] and decreasing P_{50} values, effectively. Our experience with 02-50 indicates that LR16 can be substituted with larger, bulky substituents. In the case of 02-50, two bulky trifluoromethyl groups result in a marked increase in drug effectiveness at modulating LEH P_{50}. Table I summarizes the effect of the allosteric modifiers of interest on the P_{50} value of purified hemoglobin stripped of its natural allosteric effector 2,3-DPG. Drug concentrations of 1.5 mM were employed. P_{50} refers to the partial

oxygen pressure at which purified hemoglobin solution is half-saturated in the presence of 1.5 mM drug; P_{50C} refers to the partial oxygen pressure at which purified hemoglobin solution is half-saturated in the absence of drug. The P_{50C} values ranged from 5 mm Hg to 10 mm Hg; the observed variance existing due to differences in the level of purity of hemoglobin achieved for different preparations. LEH plus 0.75 mM drug yielded a P_{50} value of 32 mm Hg. The addition of HSA concentrations of 5 mg/ml and 15 mg/ml to the LEH-drug suspension reduced the observed P_{50} values to 13 mm Hg and 11 mm Hg, respectively. The control value for LEH only (i.e. no drug or HSA) was 10 mm Hg. Table II and III summarize the effect of HSA on the ability of LR-16 to modulate the oxygen binding properties of purified human hemoglobin and liposome-encapsulated hemoglobin, respectively.

It is clear that the presence of human serum albumin (HSA) strongly modulated the pharmacological properties of LR16 and 02-50 agents. 1 mM LR16 shifts the P_{50} of hemoglobin free in solution from 8 mm Hg to a value of 49 mm Hg; however, the addition of physiologically relevant concentrations of 50 mg/ml HSA right shift the oxygen dissociation profile of hemoglobin to control P_{50} values (8 mm Hg). Strong modulation was observed for both lipid-encapsulated hemoglobin and unencapsulated hemoglobin. The effects of LR16 and 02-50 in liposome-encapsulated hemoglobin suspensions were significantly reduced by the presence of HSA. Differenetial scanning calorimetric sudies (Figures 1 and 2) demonstrate that both LR-16 and 02-50 readily interact with lipid bilayers and thus have a means to readily diffuse from the aqueous core of the liposome. When the liposome is surrrounded by HSA as is the case in human blood, the lipophilicity of the agents promote their diffusion from the particle.

HSA is well known to have two highly specific binding sites for lipophilic and negatively-charged drugs that are dependant upon the native conformation of the protein. We used a fluorescence anisotropy titration assay (Figures 3 and 4) to study the intrinsic fluorescence of camptothecin, an agent known to interact in a highly specific manner with the HSA binding pockets. We then conducted competition binding experiments to see how LR16 competes with this fluorophore for the HSA binding pockets (Figure 3). Control experiments were conducted to study how ibuprofen, an agent known to bind HSA with very high specificity, competes with the camptothecin carboxylate binding pocket on HSA (Figure 4). Whereas the well known HSA-binder ibuprofen interacted with HSA in a highly specific manner, LR-16 showed reduced competition. The reduced competition displayed by LR-16 suggests that its binding to the blood protein is much less specific or in a different site relative to the ibuprofen binding site. Experiments with denatured HSA also revealed that the albumin interactions with LR-16 were not specific in nature and that protein denaturation did not markedly attenuate drug binding. Taken together the data indicate that LR-16 binding is nonspecific and may be difficult to reduce by subtle structure changes to the drug molecule.

Molecular modeling studies (Figures 5 and 6) showed negatively-charged O2-50 fits in the deoxy-hemoglobin DPG binding pocket in between several positively charged amino acids of the two beta-chains. Several H-bonds between O2-50 and positively charged amino acids (His 2; Lys 82; His 143) of beta -chains are possible. O2-50 may stabilize the deoxy-hemoglobin by binding to the two beta-chains, thereby decreasing the oxygen affinity of hemoglobin. Future analog development studies will focus on structural changes which conserve the allosteric modulation of oxygen affinity will reducing drug associations with the HSA blood protein.

Published Abstracts/Manuscripts in Preparation

Abstracts

Demir, A., Chavan, A.J., Ostrowski, S., Priebe, W, and Burke, T.G. "Synthesis and Evaluation of Methylpropionic Acid-Derived Allosteric Modifiers of Hemoglobin", Pharmaceutical Research, 15: S-225 (1998).

Manuscripts in Preparation

Burke, T.G. et al. Synthesis and Characterization of New Methylpropionic Acid-Derived Modifiers of Human Hemoglobin Hemoglobin, manuscript in preparation.

Table I. Summary of the Effects of Allosteric Modifiers on Human Hemoglobin.[a]

Compound	P_{50}/P_{50C}
LR16	5.3
02-50	6.2

[a] Drug and hemoglobin concentrations of 1.5 mM and 150 μM, respectively, were used. Experiments were conducted at 37 °C in TES buffer, pH 7.4. P_{50} values represent determinations in the presence of drug, while P_{50C} values represent determinations in the absence of drug. P_{50C} values ranged between 5 mm Hg and 10 mm Hg, depending on how efficiently the hemoglobin was stripped of its natural allosteric effector 2,3-DPG.

Table II: Effect of HSA on the Ability of LR16 to Modulate the P_{50} Value of Human Hemoglobin in Solution.

[LR16] (M)	[HSA] (mg/mL)	P_{50} (mm/Hg)
$2x10^{-3}$	10	33.7
$2x10^{-3}$	10	33.1
$2x10^{-3}$	15	24.0
$2x10^{-3}$	15	23.9
$2x10^{-3}$	20	17.3
$2x10^{-3}$	30	10.5
$2x10^{-3}$	40	8.0
$2x10^{-3}$	50	8.0
0	0	6.0
0	50	6.0

[a] Drug and hemoglobin concentrations of 2.0 mM and 150 μM, respectively, were used. Experiments were conducted at 37 °C in TES buffer, pH 7.4.

Table III. Effect of HSA on the P_{50} for LEH/LR16 Formulations.[a]

[HSA] (mg/mL)	[LR16] (M)	P_{50} (mm/Hg)
0	$1x10^{-3}$	49.4
5	$1x10^{-3}$	34.1
15	$1x10^{-3}$	15.6
20	$1x10^{-3}$	11.5
30	$1x10^{-3}$	9.0
40	$1x10^{-3}$	8.5

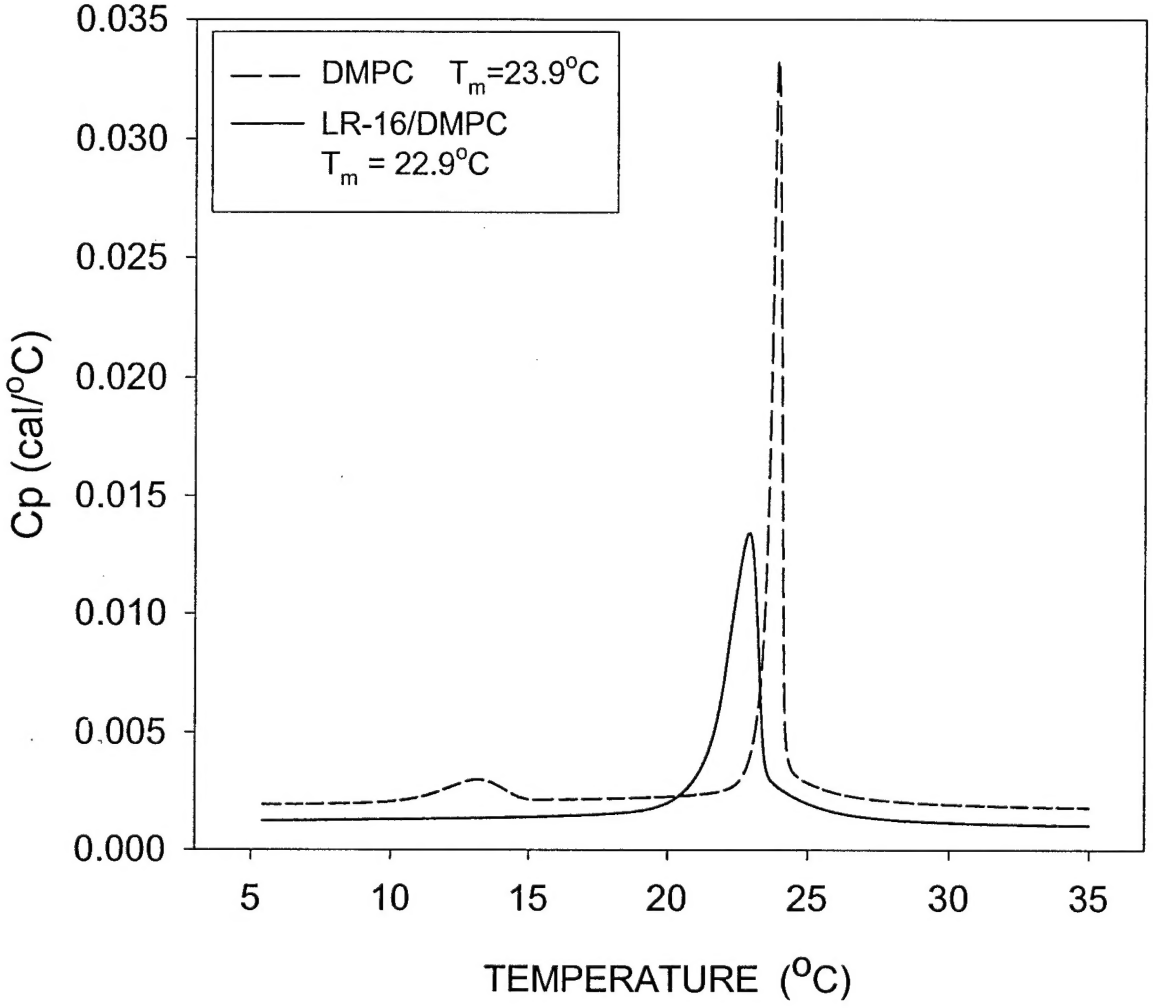


Figure 1. DSC scans showing the pronounced effect of the allosteric modifier LR-16 on the phase transition of the DMPC lipid.

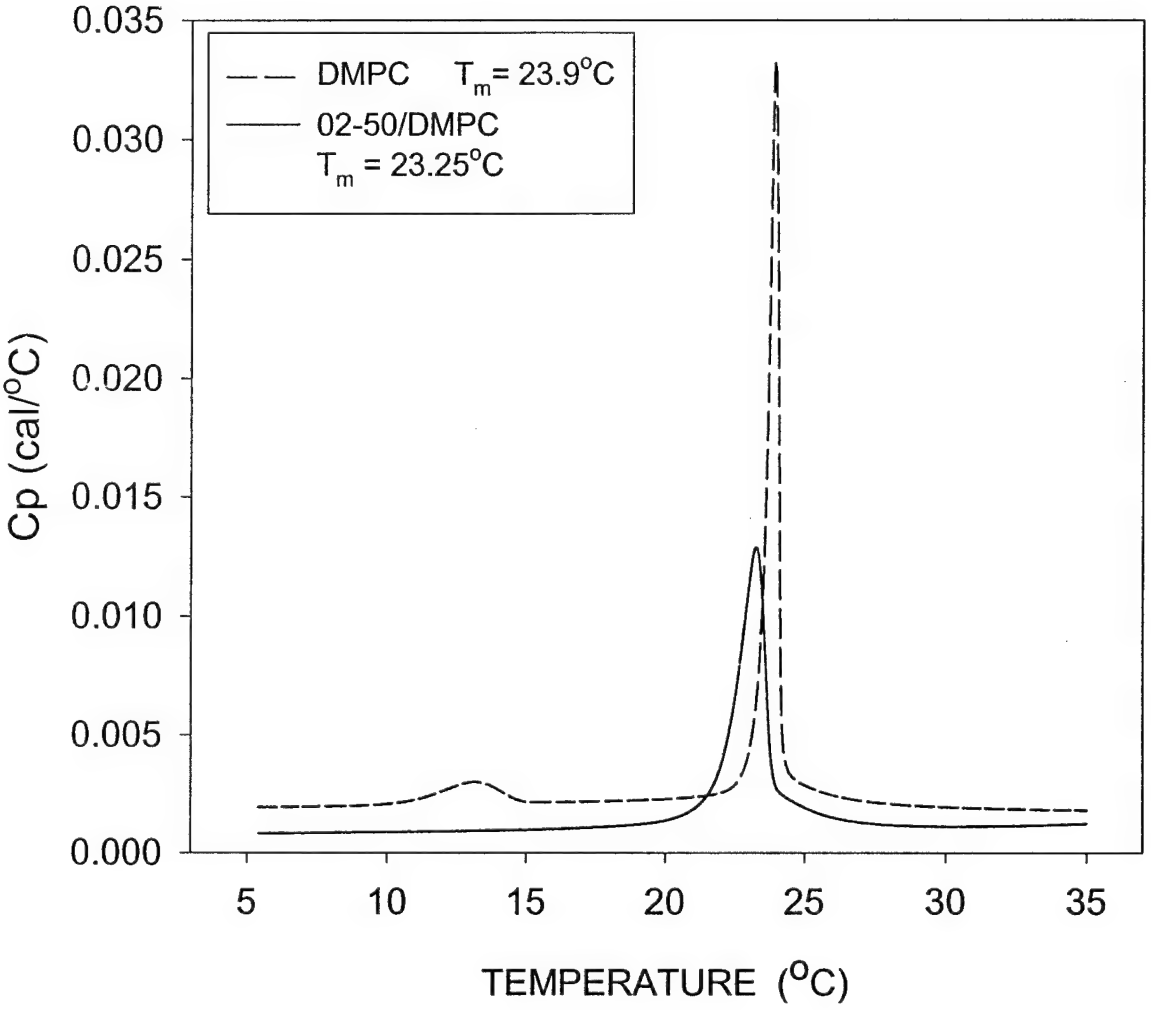


Figure 2. DSC scans showing the pronounced effect of the allosteric modifier 02-50 on the phase transition of the DMPC lipid.

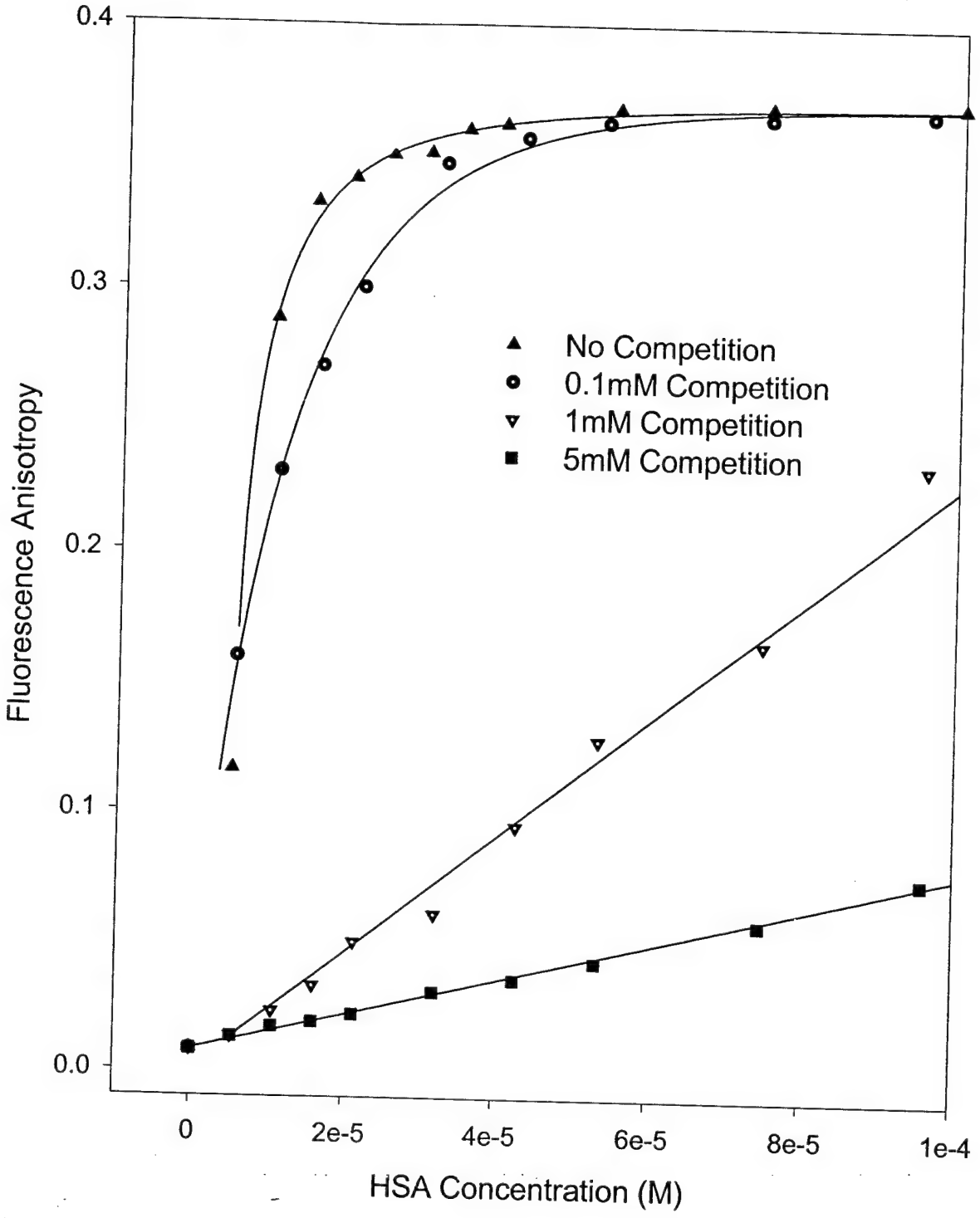


Figure 3. Fluorescence anisotropy titration profiles showing the strong interactions of camptothecin carboxylate with human albumin. Note the high increase in camptothecin carboxylate anisotropy (due to slowing of the fluorescent compound) with increasing albumin concentration. Also note that LR-16 can compete for the albumin binding pockets as evidenced by the altered anisotropy titration profile, but this competition is not nearly as specific as observed in the case of ibuprofen (Figure 4).

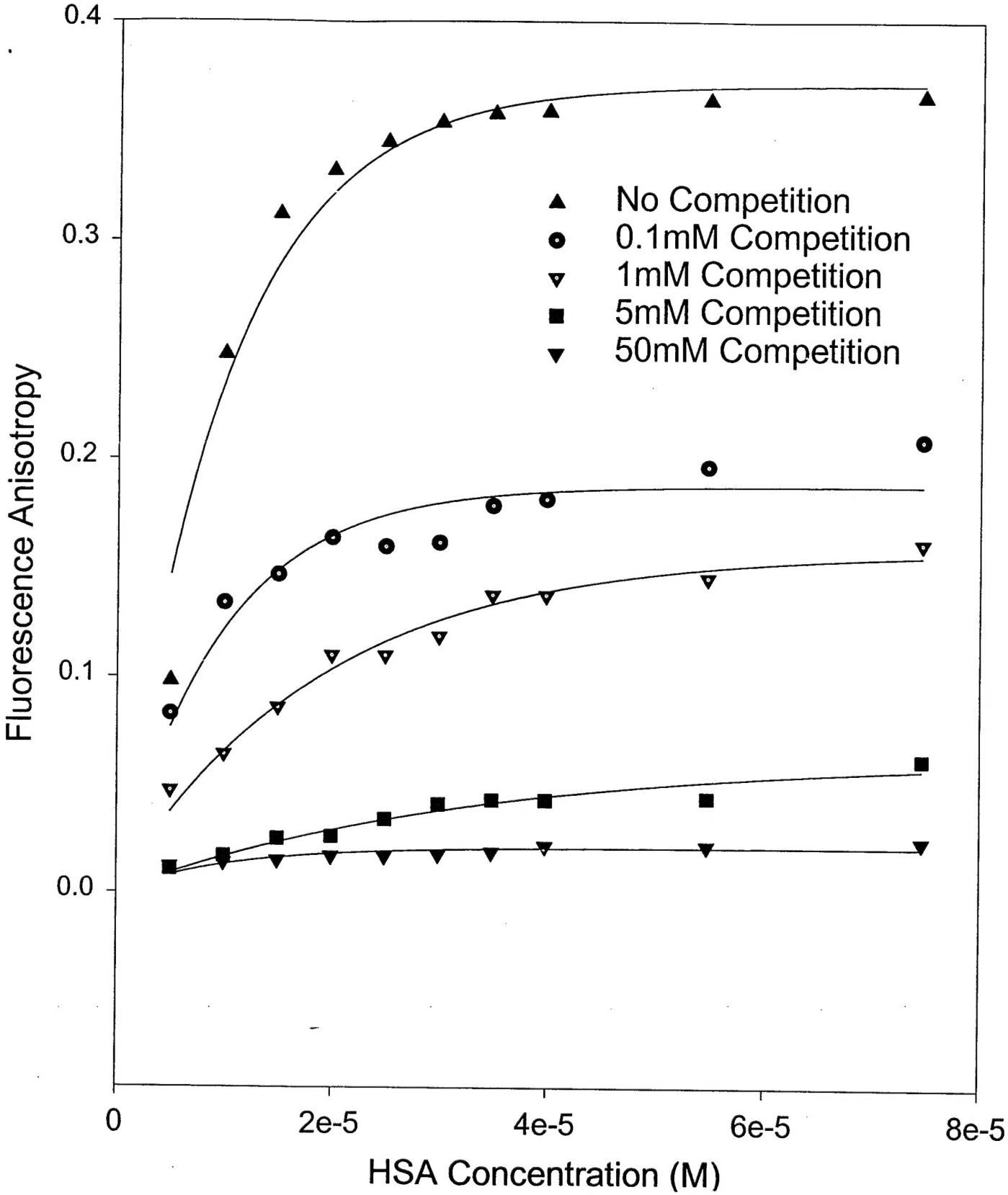


Figure 4. Fluorescence anisotropy titration profiles showing the strong interactions of camptothecin carboxylate with human albumin. Note the high increase in camptothecin carboxylate anisotropy (due to slowing of the fluorescent compound) with increasing albumin concentration. Also note that ibuprofen can compete for the albumin binding pockets as evidenced by the altered anisotropy titration profile, and that ibuprofen is very competitive at modulating camptothecin carboxylate binding.

Figures 5 and 6. Molecular modeling studies showing negatively-charged O2-50 docked with deoxy-hemoglobin. 02-50 appears in the DPG binding pocket in between several positively charged amino acids of the two beta-chains. Figure 5 shows van der Waals interactions between atoms, while Figure 6 shows a ribbon structure for the protein. The model indicates several H-bonds between O2-50 and positively charged amino acids (His 2; Lys 82; His 143) of beta -chains are possible.